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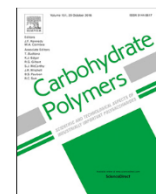
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Low methyl-esterified pectin protects pancreatic β -cells against diabetes-induced oxidative and inflammatory stress via galectin-3

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ABSTRACT

Insufficient intake of dietary fibers in Western societies is considered a major contributing factor in the high incidence rates of diabetes. The dietary fiber pectin has been suggested to be beneficial for management of both Diabetes Type 1 and Type 2, but mechanisms and effects of pectin on insulin producing pancreatic β -cells are unknown. Our study aimed to determine the effects of lemon pectins with different degree of methyl-esterification (DM) on β -cells under oxidative (streptozotocin) and inflammatory (cytokine) stress and to elucidate the underlying rescuing mechanisms, including effects on galectin-3. We found that specific pectins had rescuing effects on toxin and cytokine induced stress on β -cells but effects depended on the pectin concentration and DM-value. Protection was more pronounced with low DM5 pectin and was enhanced with higher pectin-concentrations. Our findings show that specific pectins might prevent diabetes by making insulin producing β -cells less susceptible for stress.

1. Introduction

Pancreatic islet inflammation is the main pathophysiological features of Type 1 Diabetes and late-period Type 2 Diabetes (Sudhakar et al., 2018). β -cells possess an active oxidative metabolism and a low antioxidant enzyme content (Gerber & Rutter, 2017). Therefore, they are susceptible to damage by oxidative and nitrosative stress (Gerber & Rutter, 2017). This stress is caused by overproduction of free radical species such as reactive oxygen species (ROS) and nitric oxide (NO) and is involved in induction of β -cell apoptosis (Dabhi & Mistry, 2015; Fujimaki et al., 2015). Also, during progression of the autoimmunity causing Type 1 Diabetes, invading immune cells and secretion of cytokines by those cells also provoke islet-inflammation and apoptosis by ROS and NO overproduction (Merriman & Fu, 2019).

Recently, a high pectin diet has been suggested to be effective for diabetes management (J. Wu et al., 2017). Most of these beneficial effects are attributed to altering glucose tolerance (García-Carrizo, Picó, Rodríguez, & Palou, 2019; Samout et al., 2016). However, it has not been investigated whether pectins can also directly impact β -cells. Pectin is a heteropolysaccharide dietary fiber that is isolated from cell walls of terrestrial plants (Dranca & Oroian, 2018) and can be taken up

in blood (De Leoz et al., 2013; Eiwegger et al., 2010; Hong et al., 2004; Porporatto, Bianco, & Correa, 2005). Pectins derived from lemon are mainly composed of a backbone of α -1,4-linked-D-galacturonic acid residues that are partly methyl-esterified (Moreira et al., 2014). The percentage of methyl-esterification, known as degree of methyl-esterification (DM), impacts function of several biological processes (Eliaz & Raz, 2019; Samout et al., 2016). However, the role of DM and the exact molecular mechanism behind the effects on islet survival have not been investigated.

Pectin is a natural and specific inhibitor of galectin-3 (Gal-3) (Zhang et al., 2016). Gal-3, a β -galactoside-binding lectin, is involved in cellular communication, inflammation, and apoptosis (Sehrawat & Kaur, 2020). Gal-3 is widely expressed in different cell types and found both intracellularly and extracellularly (Sehrawat & Kaur, 2020). Recent evidence suggests that Gal-3 is essential in development of diabetes and shows high expression in diabetic individuals (Yilmaz, Cakmak, Inan, Darcin, & Akcay, 2015). Gal-3-deficiency has shown to prevent diabetogenesis (Mensah-Brown et al., 2009; Yilmaz et al., 2015) and is highly expressed in pancreatic tissue (Sparre et al., 2002). Dietary fiber pectin may prevent pancreatic β -cell damage during oxidative and inflammatory stress depending on DM via Gal-3. To gain more insight in

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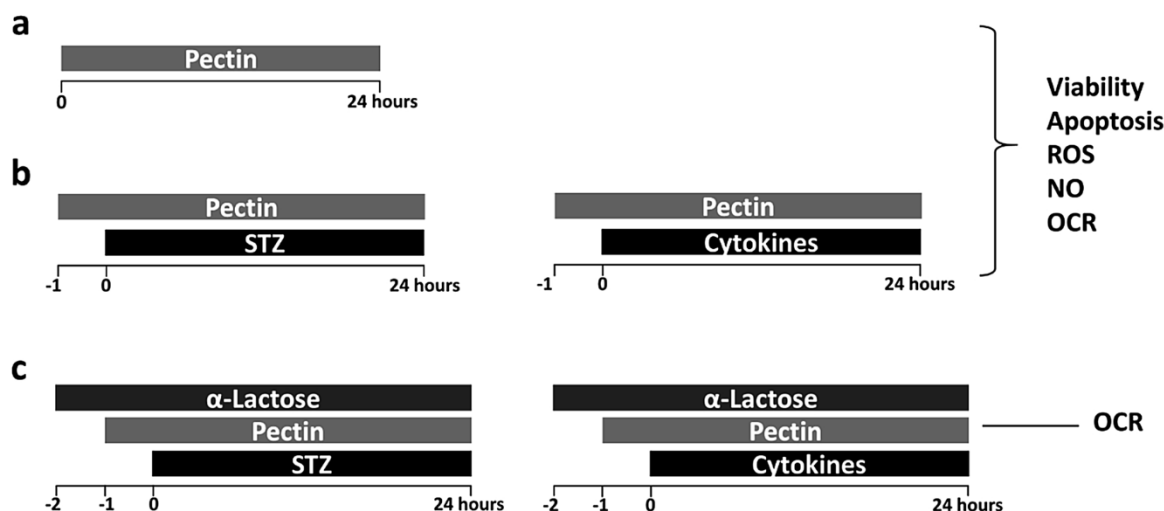


Fig. 1. Treatment schedule. Cadaveric human islets and MIN6 cells were incubated according to this treatment scheme. When adding the components (pectin, streptozotocin (STZ), α -Lactose, or cytokines) to the culture system, this was done without medium replacement. The former component was present during the following incubation.

how pectin may influence islet function, we studied the impact of pectin on human islets and a mouse β -cell line under oxidative and inflammatory stress, induced by streptozotocin (STZ) or the proinflammatory cytokines Interferon- γ (IFN- γ), Tumor necrosis factor- α (TNF- α), and Interleukin 1- β (IL-1 β). We investigated if the effects of pectin are dependent on the degree of methyl-esterification. To gain insight in a possible role of Gal-3, the Gal-3 antagonist α -lactose was applied in this study to block Gal-3 during challenge of β -cells. Our data demonstrates that pectins protect human and mouse β -cells from oxidative and inflammatory processes in a DM-dependent fashion.

2. Materials and methods

2.1. Cell culture

The mouse insulinoma MIN6 cell line (ATCC, Manassas, VA, USA) was cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 15 % fetal bovine serum (FBS, Lonza), 50 μ mol/L β -mercaptoethanol, 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 mg/L streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA) in 5% CO₂ (CO₂: O₂: N₂ = 5 : 20 : 75) at 37 °C.

2.2. Human islet isolation and culture

Human islets were isolated from cadaveric pancreata: three batches were isolated at the Leiden University Medical Center (Leiden, The Netherlands) (Ichii et al., 2005) and two batches were provided through the JDRF award 31-2008-416 (ECIT Islet for Basic Research program, Milan, Italy). Procedures were performed in accordance with the Code of Proper Secondary Use of Human Tissue in The Netherlands as formulated by the Dutch Federation of Medical Scientific Societies. After shipment, islets were handpicked and cultured in CMRL-1066 (GIBCO, Bleiswijk, the Netherlands), containing 10 % FBS, 50 U/mL penicillin, and 50 mg/L streptomycin, as previously described (Smink et al., 2016). Islets were cultured in 5% CO₂ at 37 °C.

2.3. Pectin samples

Lemon pectins with DM values of DM5 and DM18 were purchased from CP Kelco (Lille Skensved, Denmark). The DM 69 pectin was purchased from Andre Pectin (Yantai, China). Endotoxin levels in pectin were determined by Limulus amoebocyte lysate assay and found to be below 0.1 μ g/L (Sahasrabudhe et al.,

2018). The molecular weight of pectin was measured using high pressure size exclusion chromatography. The DM was determined with an Ultimate 3000 high-performance liquid chromatography (HPLC) system (Thermo Scientific). The constituent monosaccharide content and composition was determined by gas chromatography as previously described (Sahasrabudhe et al., 2018). The DM value of pectin was confirmed by analyzing the release of methanol by high-performance liquid chromatography (Voragen, Schols, & Pilnik, 1986). The DM was calculated as the total mass of released methanol (mol) from per 100 mol of galacturonic acid.

2.4. Nuclear magnetic resonance spectroscopy (NMR)

DM5, DM18, and DM69 pectins (17 mg) were suspended in 0.75 mL of D₂O. All of the samples had a pH in the range 4–4.7. ¹H-, ¹³C-NMR and Heteronuclear Multiple Quantum Coherence (HMQC) spectra were recorded on a Bruker Spectrometer (600, 150.9 MHz; Mannheim, Germany) in a 5-mm tube at 80 °C using D₂O as solvent. ¹H and ¹³C chemical shifts were reported with 3-(trimethylsilyl) propionic-2,2,3,3-d₄ acid (δ 0.00 for ¹H and for ¹³C) and acetone (δ 2.22 for ¹H and δ 30.89, δ 215.94 for ¹³C) as internal reference.

2.5. Human islet and MIN6 cell treatments

To investigate the effect of pectins on healthy β -cells, MIN6 cells and human islets were incubated with lemon pectin (DM5, DM18, DM69) dissolved in culture medium at a final concentration of 0.5, 1, and 2 g/L for 24 h (Fig. 1a). To determine the influence of pectins under stress, the cells and islets were incubated with the pectins for 1 h and then exposed for 24 h to either the apoptosis-inducer streptozotocin (STZ, Sigma-Aldrich) or the proinflammatory cytokines IFN- γ , TNF- α , and IL-1 β (all from ImmunoTools, Friesoythe, Germany) (Fig. 1b). All experiments with human islets and cells were performed at 37 °C during this study. For these experiments, the cells were treated with mouse or human proinflammatory cytokines, IFN- γ (2000 U/mL), TNF- α (2000 U/mL), and IL-1 β (150 U/mL). Cell viability, apoptosis, ROS, NO, and oxygen consumption rate (OCR) were measured after exposure to the above-described conditions. Furthermore, to test whether effects are Gal-3 dependent, MIN6 cell respiratory capacity was quantified in the presence of the Gal-3 inhibitor α -lactose (Fig. 1c). For this, cells were incubated with or without α -lactose (20 mM) (Sigma-Aldrich) 1 h before pectin incubation. For all of the above treatments, components were added into the culture system without medium replacement.

2.6. Cell viability assays

To investigate the effect of pectins on β -cell viability, WST-1 was applied (Roche, Indianapolis, IN, USA). To investigate the effect of pectins on β -cell apoptosis, the cells and islets were stained by Alexa Fluor® 488 annexin V (Biolegend, San Diego, CA, USA) and propidium iodide (PI, Thermo Scientific, Eugene, OA, USA). For staining, islets were fixed for 15 min with 4% (w/v) paraformaldehyde (Merck, Darmstadt, Germany). Immunofluorescence staining of insulin was performed as described previously (Liu et al., 2019). See supplementary methods for details.

2.7. Oxidative stress assays

Intracellular ROS was detected according to the manufacturer's instruction of a Cellular ROS Assay Kit (Abcam, Cambridge, UK). The NO concentration in the supernatants was measured with a Nitric Oxide Assay Kit (Invitrogen, Vienna, Austria) according to manufacturer's instructions. See supplementary methods for details.

2.8. Oxygen consumption analysis

The effect of pectins on mitochondrial function was measured by the Agilent Seahorse XF24 Extracellular Flux Assay Kit (Seahorse Bioscience, North Billerica, MA, USA). MIN6 cells (4×10^4 cells/well) were seeded in XF24 cell culture microplates (Seahorse Bioscience) and treated with pectin, STZ, or cytokines in the presence and absence of α -lactose. See supplementary methods for details.

2.9. Statistical analysis

Parametric distribution of data was confirmed using Kolmogorov-Smirnov tests. Data are expressed as mean \pm standard error of mean (SEM). Statistical differences of parametric data were analyzed using one-way ANOVA, while nonparametric data were analyzed with a Kruskal-Wallis test. P-values < 0.05 were considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). The data were analyzed using GraphPad Prism (version 7.00; GraphPad Software Inc, La Jolla, CA, USA).

3. Results

3.1. Structural characterization of pectins

The structural characterization of pectin DM5, DM18, and DM69 reported in Table 1 was confirmed by NMR analysis (Figs. S1–S8). The major carbohydrate residue α -linked galacturonic acid (α -GalA) and the degree of methylation was determined. The ^1H -NMR spectrum of DM5 showed the following α -GalA peaks: H-1 at 5.09, H-2 at 3.76, H-3 at 3.98, H-4 at 4.44, and H-5 at 4.77 ppm. ^{13}C -NMR of DM5 presented the following α -GalA peaks: C-1 at 99.94, C-2 at 69.06, C-3 at 69.64, C-4 at 71.81, and C-5 at 78.91 ppm. The peak at 174.71 ppm is assigned to the carbonyl group (C6). No significant OCH_3 signal was observed. The

Table 1
Structural characteristics of the pectins. Degree of methyl-esterification (DM) is defined as the amount of methanol (mole) per 100 mol of the total galacturonic acid in the sample. Molecular weight = Mw. Rhamnose = Rha, arabinose = Ara, galactose = Gal, glucose = Glc and Uronic acid = UA.

Pectin	Mw (KDa)	Monosaccharide content (mol%)					Carbohydrate content (%)
		Rha	Ara	Gal	Glc	UA	
DM5	36	0	0	3	0	95	68
DM18	52	0	0	3	1	95	73
DM69	8	0	0	8	1	87	83

^1H -NMR of DM18 showed the following α -GalA peaks: H-1 at 5.10, H-2 at 3.78, H-3 at 3.99, H-4 at 4.45, and H-5 at 4.82 ppm. The peak at 3.82 ppm can be assigned to the OCH_3 group. The integration of the OCH_3 group corresponds to $\sim 20\%$ methylation. The ^{13}C -NMR of DM18 showed peaks of α -GalA: C-1 at 100.03, C-2 at 69.03, C-3 at 69.51, C-4 at 71.66, and C-5 at 78.89 ppm. The peak at 53.52 ppm belongs to the OCH_3 group. The ^1H -NMR of DM69 that can be assigned to α -GalA: H-1 and H-5 appear as a multiplet at 5.05 ppm, H-2 at 3.69, H-3 at 3.98, and H-4 at 4.46 ppm. The OCH_3 peak resides at 3.82 ppm and its integration suggests $\sim 61\%$ methylation. However, significant overlap of the methyl peak in the ^1H spectrum prevents accurate determination of methylation. The poor solubility and high gelling capacity of DM69 prevented the acquisition of a detailed ^{13}C NMR spectrum, so the ^{13}C chemical shifts were revealed by HMQC.

3.2. Protective effect under STZ-induced stress

We first investigated the effects of pectins on human islets without any stressor. Pectins did not significantly influence cell viability with any of the tested DM-value (DM5, DM18 and DM69) or at any concentration (0.5, 1, and 2 g/L) (Fig. 2a, b). To investigate effects of pectin on islets under stress we first tested the impact of pectins on islet-cells exposed to STZ, i.e. a well-known β -cell apoptosis inducer (Biswas, Gupta, Verma, & Singh, 2017). Human islets were incubated with pectins with DM5, DM18, and DM69 and at concentrations of 0.5, 1, and 2 g/L for 1 h. Subsequently 5 mM STZ was added and incubated for 24 h.

STZ significantly reduced viability by $53.8 \pm 8.6\%$ ($p < 0.001$; Fig. 2c). This reduction was less when islets were pre-incubated with pectins. DM5 pectin showed the most pronounced protection. It prevented the STZ-induced viability decrease by $38.7 \pm 10.5\%$ ($p < 0.01$) at a concentration of 1 g/L but not at 0.5 g/L. DM5 pectin exposure at a concentration of 2 g/L showed even a stronger protective effect and prevented the decrease in viability after STZ exposure by $46.3 \pm 13.7\%$ ($p < 0.01$). Effects were still present but less pronounced with DM18 pectin which only at 2 g/L significantly prevented STZ-induced reduction in viability by $41.8 \pm 9.7\%$ ($p < 0.01$). Pectin of higher DM, i.e., DM 69, did not show any significant protective effects. The effect of pectins on islet apoptosis was also investigated. STZ at a concentration of 5 mM significantly increased islet apoptosis with $54.8 \pm 7.5\%$ ($p < 0.001$) compared to the untreated islets (Fig. 2d, e). DM5 pectin at a concentration of 2 g/L significantly prevented STZ-induced apoptosis with $46.0 \pm 8.5\%$ ($p < 0.001$). This effect was not observed when islets were pre-incubated with pectin of higher DM or lower concentrations.

Islets are clusters of several different cell types (Cabrera et al., 2006). To confirm relevance of our findings for β -cells we also performed the above-described experiments with a pure β -cell line. Since there is no human β -cell line available that responds to glucose stimulation like in healthy individuals, we used the mouse MIN6 cell line that does respond to glucose changes (Hastoy et al., 2018; Ishihara et al., 1993). As in islets, pectin alone did not show any effects on β -cell viability under homeostatic culture conditions (Fig. 2f). However, STZ significantly decreased cell viability by $53.0 \pm 1.1\%$ ($p < 0.001$) (Fig. 2g), which was significantly prevented by DM5 pectin at 1 g/L by $33.8 \pm 6.1\%$ ($p < 0.05$). DM5 pectin exposure at 2 g/L showed a stronger protective effect and prevented the decline by $40.5 \pm 2.0\%$ ($p < 0.001$). DM18 pectin at 2 g/L significantly inhibited the decrease by $27.5 \pm 2.3\%$ ($p < 0.05$). DM69 pectin did not have a significant effect here. STZ-induced apoptosis in $61.2 \pm 6.8\%$ of the MIN6 cells ($p < 0.001$; Fig. 2h). DM5 pectin at a concentration of 1 g/L and 2 g/L significantly prevented STZ-induced cell apoptosis by respectively $46.8 \pm 8.5\%$ ($p < 0.01$) and $53.4 \pm 7.3\%$ ($p < 0.001$). DM18 pectin at 2 g/L also significantly inhibited apoptosis by $45.5 \pm 7.6\%$ ($p < 0.01$). There was no rescuing effect of DM69 pectin.

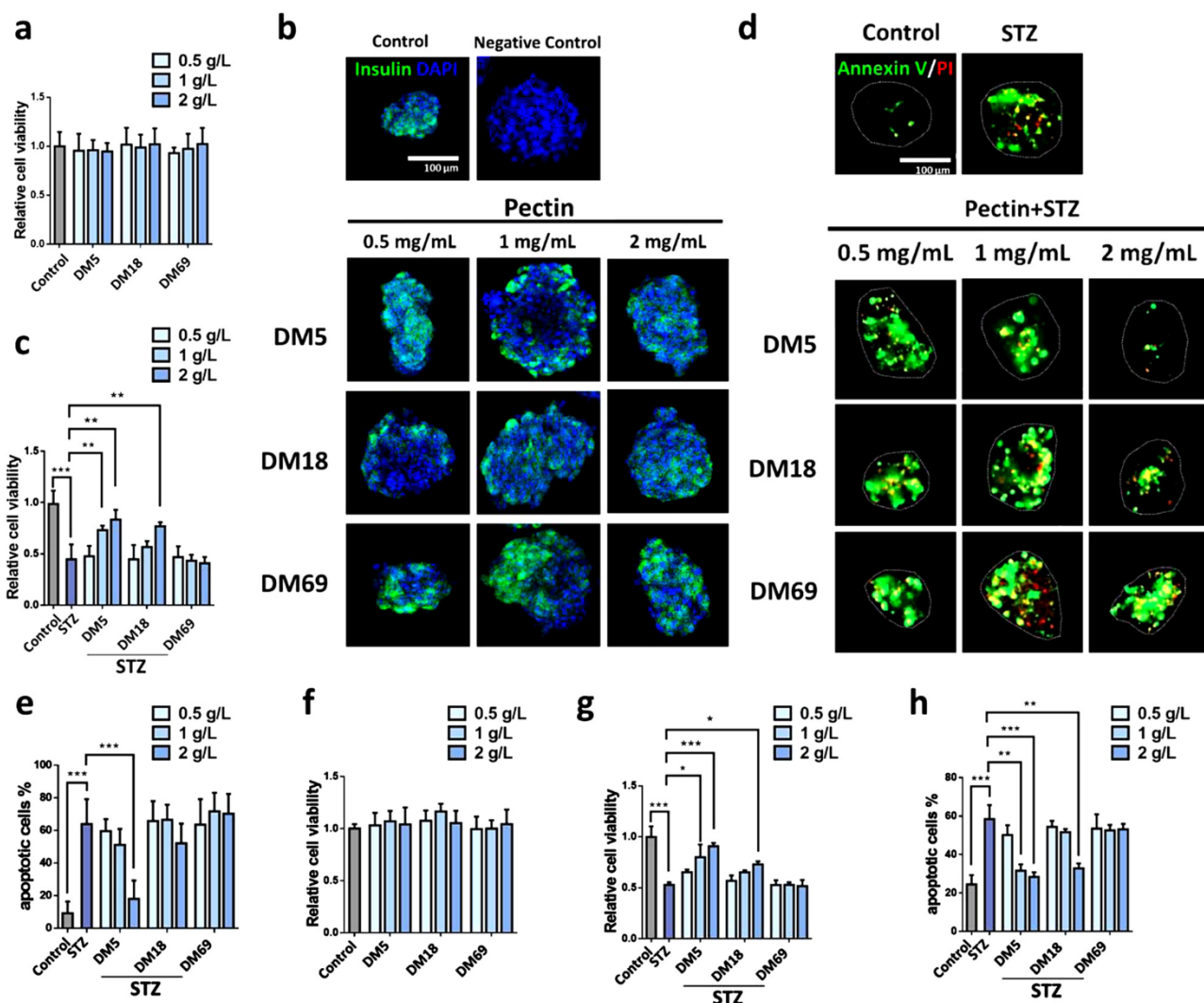


Fig. 2. Effects of pectins with different DM (DM5, DM18, and DM69) on cell survival in human islets and MIN6 cells exposure to STZ. Human islets (a and b) or MIN6 cells (f) were incubated with pectin followed by measuring cell viability (a and f) using a WST-1 assay and insulin staining (b). To investigate the effect of pectins on islet β -cell under streptozotocin (STZ)-induced stress, islets (c-e) or MIN6 cells (f-h) were incubated with pectins for 1 h followed by co-incubation for 24 h with 5 mM STZ and pectin. After incubation cell viability was determined by a WST-1 assay (c and g). Islet cell apoptosis was determined by co-staining with Annexin V and PI, and analyzed under a fluorescence microscope (d). Islet Annexin PI staining results were analyzed by using Image J gradation analysis (e). MIN6 cell apoptosis was detected using a flow cytometric assay with Annexin V and PI staining (h). Results are plotted as mean \pm SEM ($n = 5$). The statistical differences were quantified using one-way ANOVA analysis with Newman-Keuls multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Scale bar denotes 100 μ m.

3.3. Protective effect under inflammatory stress

To determine the role of pectins on β -cell survival under cytokine stress we tested the effects after exposure to the cocktail of IL-1 β + IFN- γ + TNF- α , which has been identified as essential effector molecules in the initiation of Type 1 Diabetes (van der Torren et al., 2016). The cytokines significantly decreased human islet viability with 63.2 ± 8.3 % ($p < 0.001$; Fig. 3a). However, a pre-incubation with pectin prevented this viability decrease. DM5 at a concentration of 1 g/L significantly prevented the viability decline with 45.7 ± 8.0 % ($p < 0.001$) and at 2 g/L almost completely prevented the negative effects on viability. Effects were less at higher DM as DM18 pectin only prevented cytokine damage at 2 g/L significantly with 43.8 ± 10.9 % ($p < 0.01$) and not at lower concentrations. DM69 pectin did not influence islet viability after cytokine exposure. Inflammatory cytokine-induced islet apoptosis is the main cause of islet loss (Arroyo-Jousse, Garcia-Diaz, Codner, & Pérez-Bravo, 2016). Therefore, apoptosis was investigated following incubation with pectins and cytokines. Cytokines significantly increased apoptosis by 77.2 ± 7.6 % ($p < 0.001$) of the human islet-

cells (Fig. 3b, c). However, the apoptosis was significantly reduced with 43.9 ± 5.8 % ($p < 0.001$) when islets were pre-incubated with DM5 pectin at 2 g/L. Pectins of DM18 and DM69 did not show an effect on apoptosis at any concentrations.

We also studied the impact of cytokines and pectins on MIN6 cells. Cytokine-treated cells showed a decrease in cell viability of 57.9 ± 2.6 % ($p < 0.001$) compared to controls. This decrease was prevented by low-DM pectins (Fig. 3d). DM5 pectin at 1 g/L significantly prevented the viability decrease with 44.0 ± 5.4 % ($p < 0.01$). When further increasing the dosages of DM5 pectin to 2 g/L, the pectin almost completely prevented the negative effect on viability ($p < 0.001$). DM18 pectin at 2 g/L demonstrated a protective effect but less effective as DM5 pectin. It prevented decrease in cell viability with 27.8 ± 4.0 % ($p < 0.05$). DM69 pectin-pretreated cells did not show significant differences. To also determine the influence of pectin on cytokine-induced β -cell apoptosis, we measured apoptosis of MIN6 cells incubated with cytokines in presence and absence of pectin. Cytokines increased apoptosis by 77.2 ± 7.6 % ($p < 0.001$; Fig. 3e). DM5 pectin at a concentration of 1 g/L and 2 g/L significantly prevented this increase

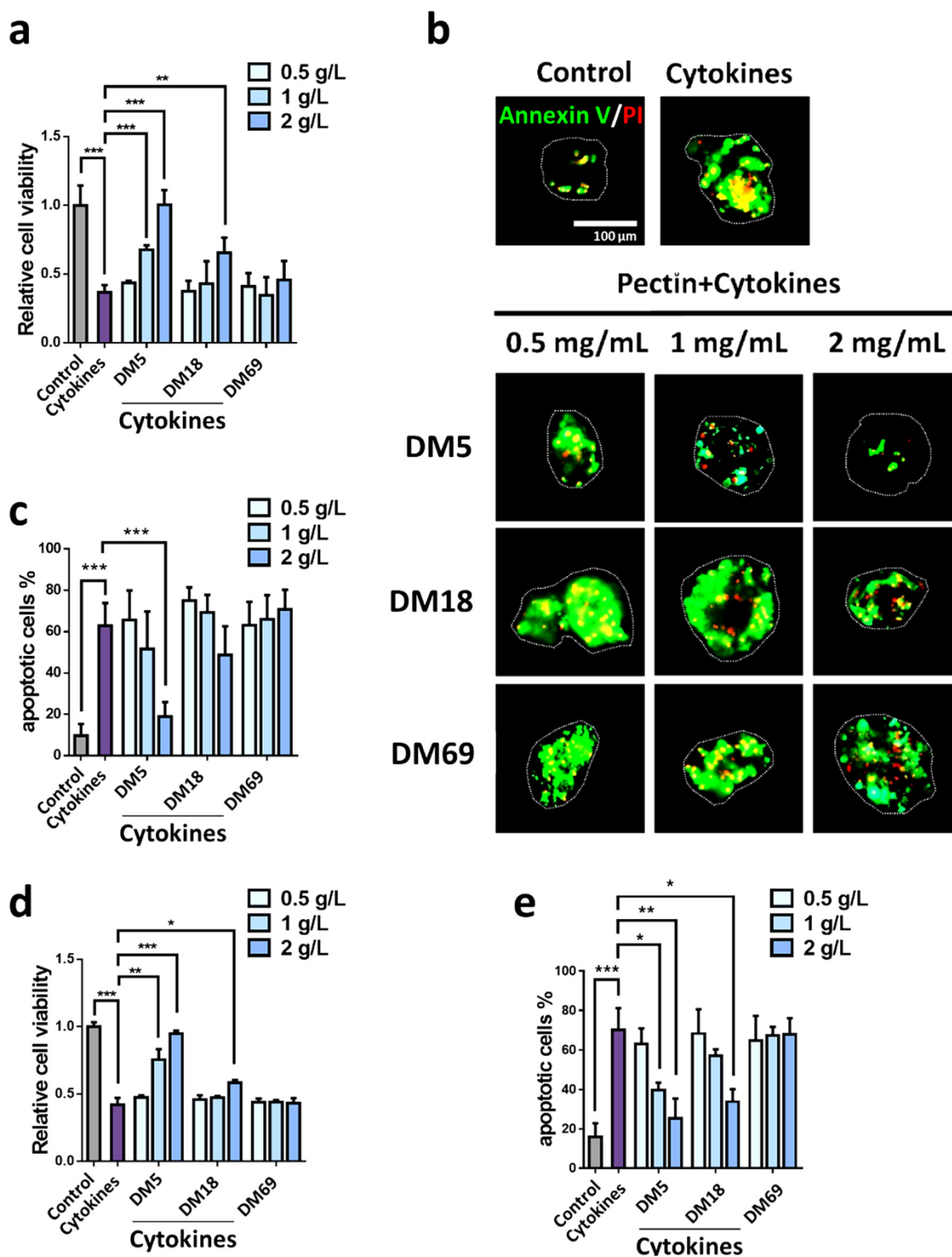


Fig. 3. Effects of DM5, DM18, or DM69 pectin on cell survival of human islets and MIN6 cells exposure to proinflammatory IFN- γ , TNF- α , and IL-1 β cytokine cocktail. Human islets (a-c) and MIN6 cells (d and e) were incubated with pectins for 1 h followed by co-incubated of pectins and cytokines (IFN- γ , TNF- α , and IL-1 β) for an additional 24 h. After incubation, cell viability was determined by a WST-1 assay (a and d). Cell apoptosis was determined by co-staining with Annexin V and PI. Human islets were imaged with a fluorescence microscope (b) and analyzed by using Image J gradation analysis (c). MIN6 cell apoptosis was detected using a flow cytometric assay (e). Results are plotted as mean \pm SEM (n = 5). The statistical differences were analyzed using one-way ANOVA analysis with Newman-Keuls multiple comparisons test. (* p < 0.5, ** p < 0.01, *** p < 0.001). Scale bar denotes 100 μ m.

with 43.4 ± 8.3 % (p < 0.05) and 63.7 ± 8.6 % (p < 0.01). DM18 pectin only showed a rescuing effect at 2 g/L, the apoptosis was reduced with 51.9 ± 7.4 % (p < 0.01) compared to cells treated with cytokine cocktail. DM5 pectin could prevent cytokine-induced apoptosis.

3.4. Pectin attenuates generation of free radicals

As early stages of STZ-induced β -cell damage are characterized by free radical generation, we studied whether pectins impact the release

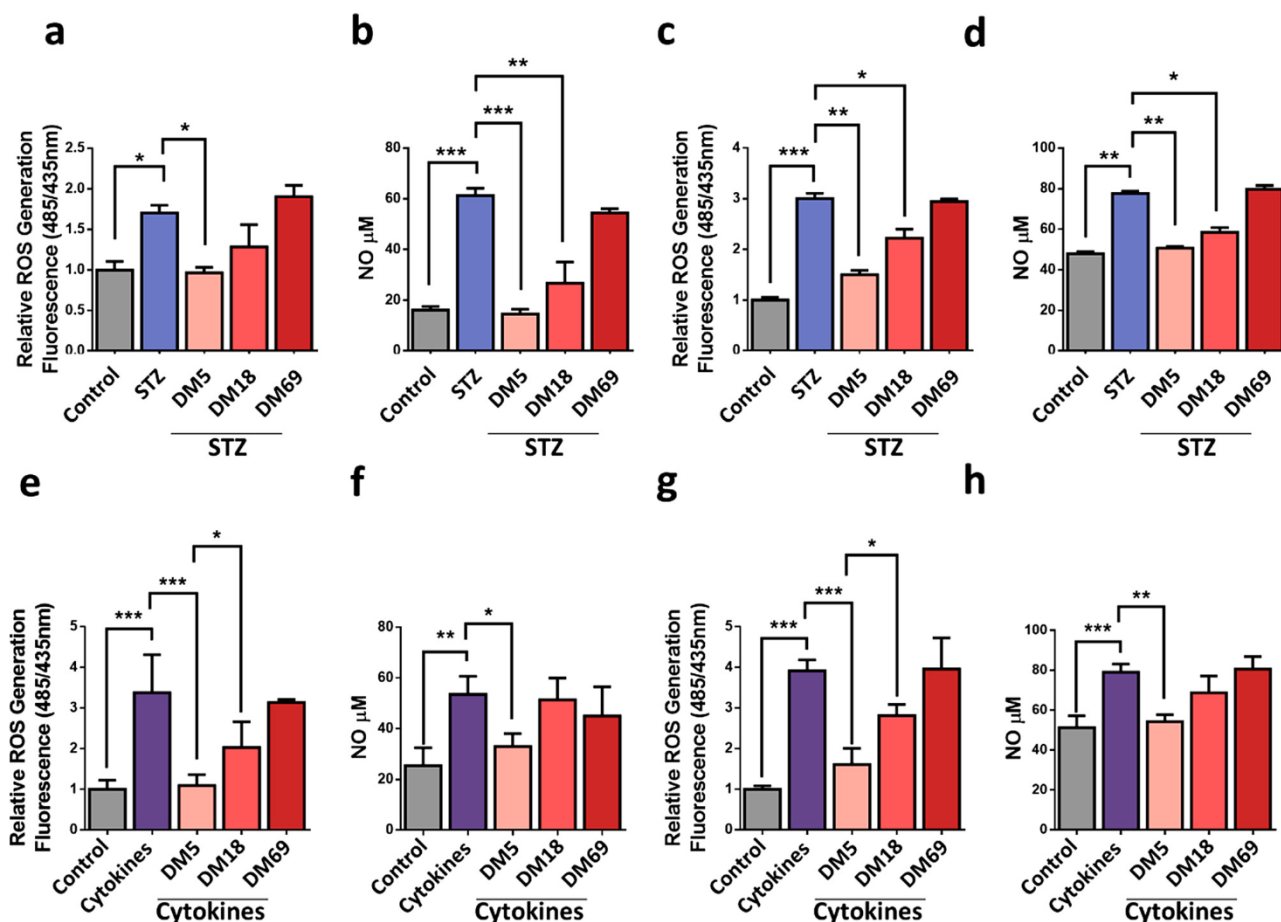


Fig. 4. Effects of DM5, DM18, or DM69 pectin on STZ- or cytokine-induced free radical generation in human islets and MIN6 cells. Human islets (a, b, e and f) or MIN6 cells (c, d, g and h) were incubated with 2 g/L pectin for 1 h followed by 24 h co-incubation with pectin and 5 mM STZ (a-d) or a cytokine cocktail (e-h). Intracellular ROS was measured with a DCFDA Cellular ROS Detection Assay Kit (a, c, e and g). Nitric oxide (NO) was detected using a Nitric Oxide Assay Kit (b, d, f and h). Results represent are expressed as mean \pm SEM (n = 5). The statistical differences were analyzed using one-way ANOVA analysis with Newman-Keuls multiple comparisons test. (* p < 0.05, ** p < 0.01, *** p < 0.001).

of ROS and NO (Wu et al., 2016). Since 2 g/L pectins had the most pronounced effect, these experiments were only done with this concentration. ROS generation was significantly increased with 41.4 ± 8.4 % (p < 0.05) after human islets were treated with STZ (Fig. 4a). This increase was prevented by DM5 pectin with 43.2 ± 6.8 % (p < 0.05), but not by DM18 and DM69 pectin. STZ also strongly impacted NO synthesis of islets, which was enhanced with 73.7 ± 5.3 % (p < 0.001; Fig. 4b) compared to untreated controls. DM5 and DM18 pectin prevented this enhancement with respectively 76.2 ± 5.7 % (p < 0.001) and 56.3 ± 7.7 % (p < 0.01). DM69 pectin was unable to prevent the NO overproduction.

We also studied ROS and NO generation in MIN6 cells showing that STZ provoked an increased production of ROS with 66.9 ± 4.0 % (p < 0.001) as compared with untreated controls (Fig. 4c). DM5 pectin significantly prevented this increase with 50.1 ± 4.5 % (p < 0.01) compared to cells incubated with only STZ. DM18 pectin also significantly suppressed the increase in ROS production with 26.1 ± 4.4 % (p < 0.05), but DM69 pectin did not show significant prevention. Consistent with the human islet results, STZ enhanced NO release from MIN6 cells with 38.4 ± 1.9 % (p < 0.01; Fig. 4d). DM5 pectin prevented this STZ-induced NO increase with 34.9 ± 1.9 % (p < 0.01) resulting in similar levels of NO compared to the untreated control. DM18 pectin also significantly prevented STZ-induced NO increase with 24.8 ± 1.9 % (p < 0.05), whereas DM69 pectin did not impact NO release.

cytokines, trigger β -cell death via apoptosis (Kim, Lee, Gao, & Jung, 2005). Therefore, we investigated production of ROS and NO in both islets and MIN6 cells after treatment with our cytokine cocktail and 2 g/L of the different pectins. Treatment with cytokines significantly increased ROS production in islets with 70.3 ± 12.7 % (p < 0.001; Fig. 4e). Pretreatment with DM5 pectin significantly prevented the cytokine-induced ROS generation, which was similar to untreated controls (p < 0.001). A preincubation with DM18 did also significantly reduce ROS production with 40.0 ± 14.9 % (p < 0.05). However, DM69 pectin exposure did not prevent the increase. NO was elevated after cytokine incubation with 52.5 ± 14.6 % (p < 0.01; Fig. 4f). This increase was prevented by DM5 pectin with 38.4 ± 16.4 % (p < 0.05), but not by DM18 and DM69 pectin.

In MIN6 cells we observed similar results as in the human islets. Cytokines provoked a significant increase of ROS with 74.5 ± 6.9 % (p < 0.001) as compared with untreated controls (Fig. 4g). DM5 pectin suppressed this overproduction with 58.9 ± 7.7 % (p < 0.001). DM18 pectin has less effect but still significantly suppressed the increase in ROS production with 26.1 ± 9.7 % (p < 0.05). DM69 pectin did not show a significant effect. Furthermore, cytokines enhanced NO release in MIN6 cells with 35.2 ± 6.2 % (p < 0.001; Fig. 4h). DM5 pectin prevented this NO increase with 31.4 ± 6.8 % (p < 0.01), which made the NO level similar to the untreated control. DM18 and DM69 pectin did not prevent the NO increase.

icals, induced by proinflammatory

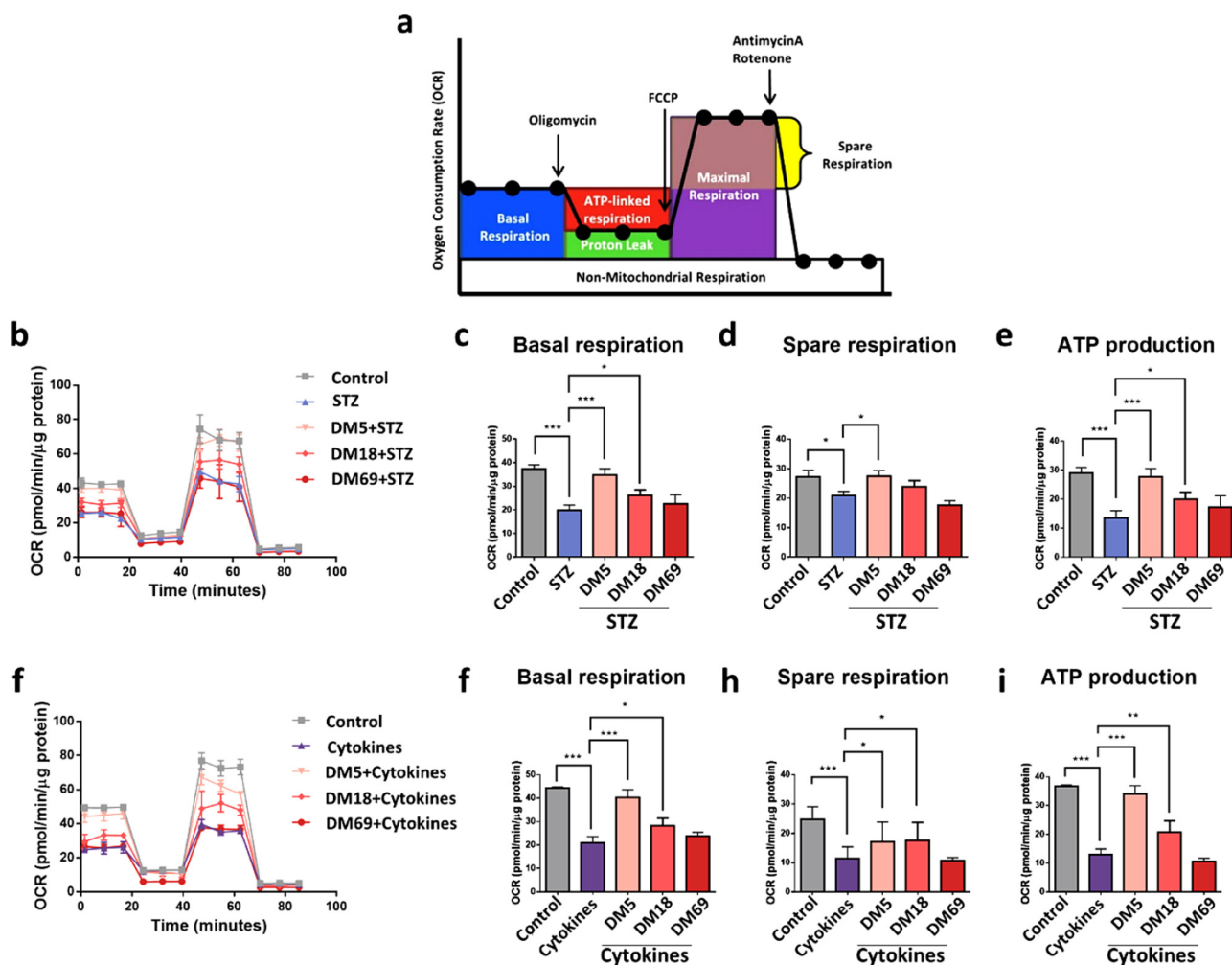


Fig. 5. Effect of DM5, DM18, or DM69 pectin on oxygen consumption rate of MIN6 cells after STZ or cytokine-induced stress. (a) A schematic overview of the mitochondrial stress test. Arrows indicate the subsequent addition of the ATPase inhibitor oligomycin, the uncoupling reagent FCCP, and the inhibitors of the electron transport chain rotenone/antimycin A. (b) MIN6 cell were seeded in a Seahorse cell culture plate. After incubation with pectins at 2 g/L for 1 h, STZ at a final concentration of 5 mM (b-e) or a cytokine cocktail (f-i) was added to each well and co-incubated with pectin for 24 h. OCR of treated cells was investigated using Seahorse Bioscience XF24 extracellular flux analyzer. Fig. c-e and g-i respectively represent individual parameters for basal respiration, spare respiration, and ATP-linked respiration. Results are plotted as mean \pm SEM ($n = 5$). The statistical differences were analyzed using one-way ANOVA analysis with Newman-Keuls multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.5. Pectin prevents damage in energy metabolism

The release of free radicals is often accompanied by mitochondrial dysfunction, which leads to disorders in the cellular energy metabolism (Banerjee et al., 2020). To investigate whether the tested pectins contribute to maintenance of β -cell energy metabolism after STZ exposure, we determined the effect of pectin on the OCR of MIN6 cells, which is an indicator of mitochondrial respiration in islet-cells (Fig. 5a) (Llacua, de Haan, & de Vos, 2018). As these assays require high cell amounts, it could only be performed with MIN6 cells and not with the rarely available human islets.

STZ has a strong negative impact on the OCR of β -cells, which was prevented by pectins in a DM-dependent fashion (Fig. 5b-e). MIN6 cells incubated with STZ showed a decreased respiration rate, including a significantly decreased basal respiration, which was 46.8 ± 2.4 % ($p < 0.001$) lower than controls, a spare respiration reduction of 23.2 ± 3.6 % ($p < 0.05$), and a reduced ATP production of 53.5 ± 3.6 % ($p < 0.001$). A pre-incubation with DM5 pectin almost completely prevented these negative effects. The mitochondrial basal respiration rate, spare respiration, and ATP-linked respiration values were similar to controls when cells were exposed to STZ. DM18 pectin had a partial rescue effect. Cells

exposed to DM18 pectin prevented the STZ-induced basal respiration decrease with 16.9 ± 2.8 % ($p < 0.05$) and ATP-linked respiration with 22.4 ± 4.0 % ($p < 0.05$). The spare respiration was still affected by STZ. DM69 pectin did not protect the cells from STZ-induced respiratory damage.

To determine the influence of pectin on cytokine-induced mitochondrial dysfunction, we treated MIN6 cells with the cytokines in the presence and absence of pectins at 2 g/L and measured OCR after 24 h. The culture of β -cells with cytokines significantly decreased OCR, it reduced the basal respiration with 52.8 ± 2.0 % ($p < 0.001$), spare respiration with 53.9 ± 8.0 % ($p < 0.001$), and ATP-linked respiration with 64.6 ± 1.8 % ($p < 0.001$; Fig. 5f-i). However, cells pre-incubated with pectins were protected from these effects. DM5 pectin significantly prevented the decrease in basal respiration with 48.1 ± 3.5 % ($p < 0.001$), spare respiration with 33.6 ± 3.2 % ($p < 0.05$), and ATP-linked respiration with 69.7 ± 5.2 % ($p < 0.001$) as compared with cells incubated with only cytokines. DM18 pectin was less efficient but still had significant protective effects. It prevented a basal respiration decrease of 25.8 ± 4.9 % ($p < 0.05$), reduced spare respiration with 35.3 ± 3.8 % ($p < 0.05$), and prevented ATP-linked respiration decline with 37.7 ± 7.0 % ($p < 0.01$). DM69 pectin did not show any significant effects here.

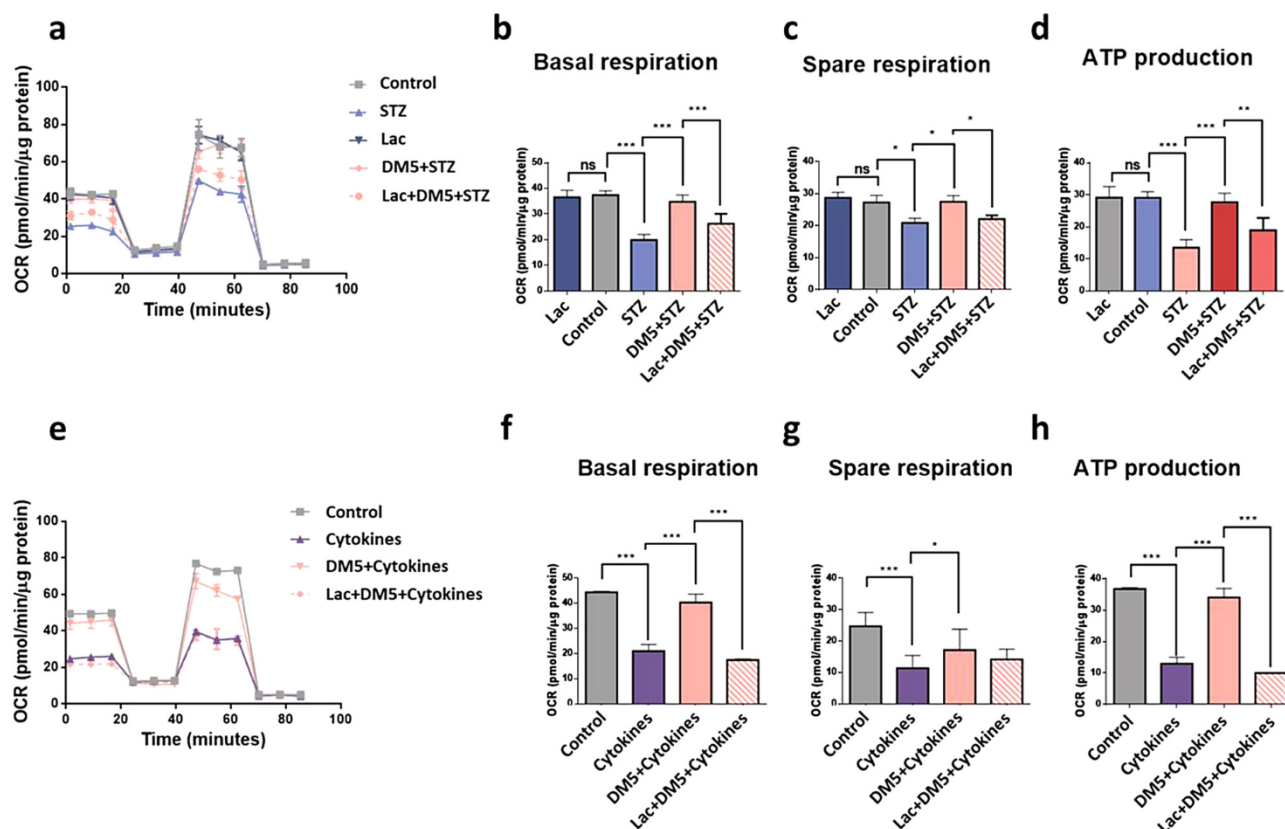


Fig. 6. DM5 pectin influences islet cell respiration through Galectin-3 (Gal-3). To verify if pectin prevents the STZ- or cytokine-induced OCR reduction through interaction with Gal-3, OCR was measured in the presence and absence of the Gal-3 ligand α -lactose (Lac). MIN6 cells were seeded in a Seahorse cell culture plate. Lactose at 20 mM was incubated with MIN6 cells for 1 h, followed by DM5 pectin and STZ (a-d) or a cytokine cocktail (e-h) for an additional 24 h. OCR of treated cells was investigated using the Seahorse Bioscience XF24 extracellular flux analyzer. Fig. b-d and f-h respectively represent individual parameters for basal respiration, spare respiration, and ATP-linked respiration. Results are plotted as mean \pm SEM ($n = 5$). The statistical differences were analyzed using one-way ANOVA analysis with Newman-Keuls multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.6. Pectins rescue damaged cells by binding to Gal-3

Pectin influences adipocyte metabolism and cancer cell apoptosis as a natural ligand of Gal-3 (Fang et al., 2018; Zhang et al., 2016). To verify if pectin prevents STZ-induced OCR reduction through interaction with Gal-3, the OCR was measured in the presence and absence of the Gal3 antagonist α -lactose (Fang et al., 2018). Since DM5 pectin showed the most pronounced effect, this experiment was only done with DM5 pectin. An incubation with α -lactose alone did not influence β -cell respiration in normal culture conditions (Fig. 6a-d). The mitochondrial basal respiration rate, spare respiration, and ATP-linked respiration of MIN6 cells exposed to α -lactose were identical to untreated controls. However, in the presence of α -lactose, the preventive effects of DM5 pectin were compromised. The preincubation with α -lactose before pectin and STZ resulted in reduced basal respiration, spare respiration, and ATP-linked respiration, which was respectively $24.4 \pm 4.4\%$ ($p < 0.01$), $19.5 \pm 8.2\%$ ($p < 0.05$), and $31.6 \pm 5.7\%$ ($p < 0.01$) lower than cells treated with pectin and STZ.

We also treated MIN6 cells with DM5 pectin and the cytokines with and without preincubation of α -lactose. A preincubation of α -lactose prevented the protective effect of DM5 pectin on cytokine-damaged β -cells (Fig. 6e). The protective effect of DM5 on basal respiration was no longer present, inclusion of the α -lactose decreased the basal respiration with $56.8 \pm 2.7\%$ ($p < 0.001$) compared with cells treated with DM5 pectin and cytokines (Fig. 6f). The preincubation of lactose did not show considerable influence on spare respiration (Fig. 6g). The rescue effect of DM5 pectin on ATP-linked respiration was fully counteracted (Fig. 6h). This indicates that DM5 pectin rescues β -cell metabolism.

4. Discussion

Type 1 and Type 2 Diabetes are frequently associated with oxidative and inflammatory stress-induced β -cell loss (Danobeitia et al., 2017; Hu, Kuwabara, de Haan, Smink, & de Vos, 2020; Usmani-Brown et al., 2019). Many report beneficial effects of pectin on glucose management, but not much is known about the direct impact on β -cells under stress (García-Carrizo et al., 2019; Wu et al., 2017). Previous studies have shown that pectin may regulate the function of Gal-3, which have been shown to participate in cytokine-induced apoptosis (Nishikawa et al., 2018). Here, we showed that pectins can rescue β -cell viability and their respiratory metabolism under STZ- or cytokine-induced stress. Furthermore, pectins reduced oxidative and nitrosative stress in both human islets and β -cells. This protective effect against STZ or inflammatory stress was dependent on the DM and the concentration of the pectins.

Previous mouse studies demonstrate a potential role for Gal-3 in anti-diabetic effect of pectins (Li et al., 2016; Mensah-Brown et al., 2009). These studies did not investigate the direct protective effects of pectin on islets. Islets are believed to be micro-organs containing several cell types (Cabrera et al., 2006; Saksida et al., 2013). By using a combination of human islets and a β -cell line, we proved the direct protective effects of pectin on β -cells. We show pectins rescue from oxidative or inflammatory stress through Gal-3. Gal-3 is synthesized by free ribosomes in the cytosol but can easily cross the plasma membrane and the endomembrane system to translocate into the nucleus, mitochondria, and extracellular matrix (Sehrawat & Kaur, 2020). Since pectin is a macromolecule and cannot access cytoplasm, pectin likely binds with Gal-3 at the surface of the cellular membrane and in the

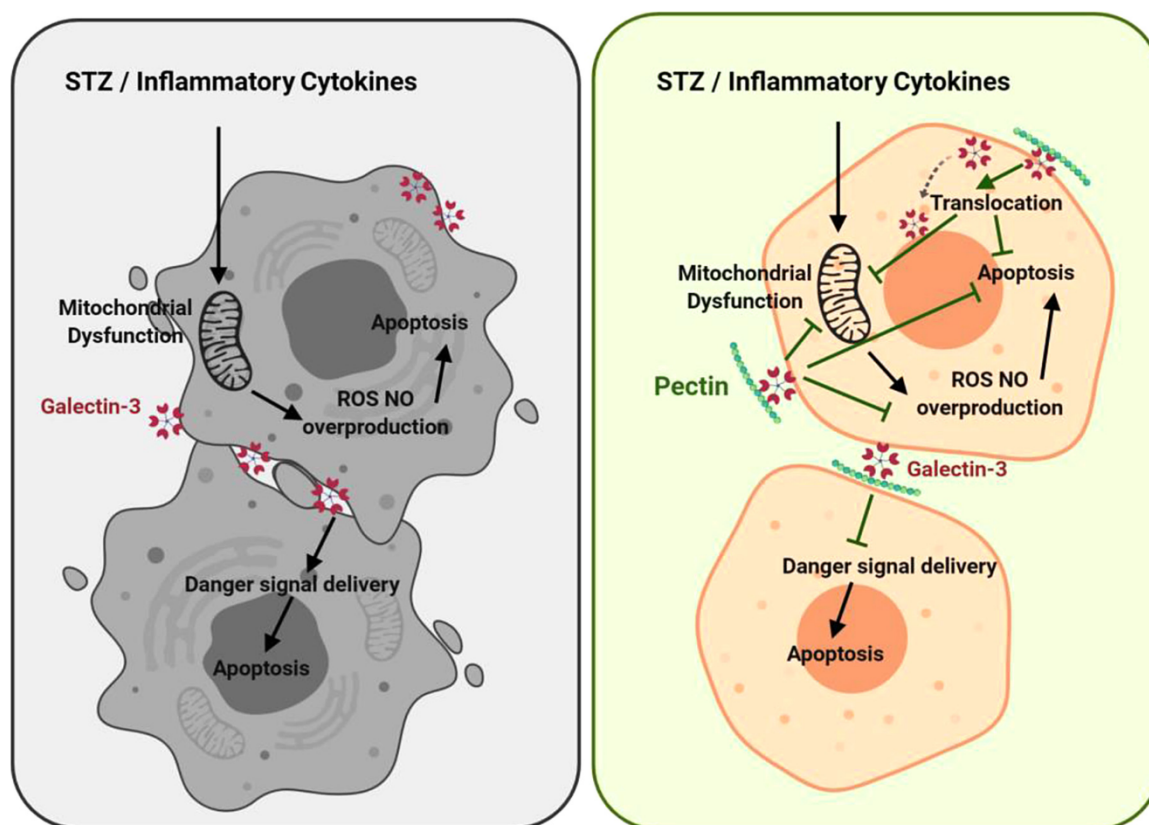


Fig. 7. Schematic illustration of the rescuing effects of low-DM pectin on β -cells. Cellular membrane and extracellular Gal-3 binding with low-DM pectin inhibits mitochondrial dysfunction, ROS and NO overproduction, and apoptosis in β -cells under STZ- or proinflammatory cytokine-induced stress.

extracellular matrix (Sahasrabudhe et al., 2018).

The mechanism behind the anti-apoptotic effects may be via three ways (Fig. 7). First, pectin is directly inhibiting pro-apoptotic Gal-3 located on the cell membrane (Fukumori et al., 2003). This binding suppresses related mitochondrial apoptotic pathways and rescues mitochondrial respiratory function (Fukumori et al., 2003). Second, pectin is hampering intracellular danger signal delivery. Gal-3 mediates the ligation of cell surface glycoproteins and increases the affinity of cell binding, which in turn facilitates intercellular signaling (Colin Hughes, 2001). The severely impaired β -cell can generate danger signals that induce nearby cell apoptosis (Paredes-Juarez et al., 2015). The binding of pectin to Gal-3 potentially suppresses danger signal delivery, subsequently improving cell survival under stress. Third, another plausible explanation could be that pectin induces translocation of Gal-3 to the perinuclear membranes (Yu, Finley, Raz, & Kim, 2002). This translocation protects mitochondrial integrity and inhibits apoptosis (Yu et al., 2002). However, the exact mechanism by which Gal-3 translocates remains subject of debate (Funasaka, Raz, & Nangia-Makker, 2014). Further research will enable us to exactly pinpoint which of the three mechanism described above is applicable to Gal-3 in β -cell apoptotic processes by pectins.

The protective effect against STZ or cytokines is dependent on pectin concentration and DM value of the pectin. The low-DM pectin at high concentration showed the highest efficiency in protection against oxidative and inflammatory damage. The DM-dependent effect may be related to the molecular weight of pectin, which can be explained by differences in the valid binding-domain densities of pectins with different DM values. Considering that pectin is a long-chain polymer, each single chain has multiple binding regions (Eliaz & Raz, 2019). Although longer chain polymers could contain more binding regions, limited by complicated structure and distance between regions, partial regions in

et al., 2016). The procedure applied for pectin de-esterification results in shorter chains and a decrease in the molecular weight of the pectin (Hotchkiss et al., 2002; Sahasrabudhe et al., 2018). This procedure may also liberate binding regions from complicated long-chain pectins and allow them to freely bind with Gal-3. Therefore, the lowest DM pectin has a maximum amount of freely binding regions per unit mass that leads to a more prominent effect (Eliaz, 2019).

The composition of sidechain monosaccharides might influence the biological characteristics of pectins (Torkova et al., 2018). Among the tested pectins in this study, similar sugar composition patterns were found in DM5 and DM18 pectin. However, DM5 pectin showed significantly stronger protection against β -cell damage. This indicates the DM values of pectins do influence the protective ability of pectin. Additionally, it was suggested that the pectin structure present a high diversity according to their different origin (Muller-Maatsch et al., 2016). Recent studies of pectins extracted from other sources, e.g. okra, sour cherry pomace, and papayas, suggest the essential roles of pectin's molecular weight and the degree of methyl-esterification on its chemical and biological characteristics (Mao et al., 2020; Prado et al., 2020). The comparisons of pectin extract from different sources may also contribute to a deeper understanding of structure-related biological function.

Our study suggests that low-DM lemon pectins, could potentially be applied in the prevention and management of diabetes by protecting β -cells against inflammatory and oxidative stress. This is, to the best of our knowledge, a new explanation as to why increased dietary fiber intake is associated with a lower frequency of hyperglycemia. Treatment of intestinal inflammation and tumors with dietary fibers showed that these polysaccharides are taken up by gastrointestinal macrophages, transported to the bone marrow, and subsequently secreted into the peripheral circulation (De Leoz et al., 2013; Eiwegger et al., 2010; Hong et al., 2004; Porporatto et al., 2005). This indicates

pectins can be blood-born, and influence tissue metabolism. The insufficient intake of dietary fibers in Western societies has been believed to be one of the major causes of the high incidence rates of both Type 1 Diabetes and Type 2 Diabetes (Mishra et al., 2019). Previous studies report beneficial effects of dietary fiber on glucose metabolism, but none of these effects is attributed to directly impacting metabolism or viability of β -cells under stress (Wu et al., 2016, 2017). Our study demonstrates that low-DM pectin plays an essential role in maintaining β -cell metabolism and promoting survival under stressful conditions. Islet transplantation, a promising treatment for Type 1 Diabetes, which is challenged by oxidative stress-induced islet graft loss, could also benefit from these results (Bottino et al., 2004; Hu & de Vos, 2019). As a natural polymer with excellent biocompatibility (Singha et al., 2017), low-DM pectin could be applied as a preincubation or coating of the islet graft to improve graft survival. Based on its biocompatibility and biodegradability, we believe that the highly abundant and low-cost natural polymer pectin might has a great potential for reducing the expense and cytotoxicity of diabetes treatment (Singha et al., 2017). In conclusion, this study provides new insights in how pectin can contribute to maintenance of health. Our data reveals an unrecognized influence of pectin on β -cell apoptosis in the oxidative and inflammatory context, showing to improve β -cell survival through binding with Gal-3.

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CRediT authorship contribution statement

Shuxian Hu: Conceptualization, Methodology, Investigation, Formal analysis, Software, Writing - original draft. **Rei Kuwabara:** Conceptualization, Methodology, Writing - review & editing. **Martin Beukema:** Conceptualization, Methodology, Writing - review & editing. **Michela Ferrari:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing. **Bart J. de Haan:** Conceptualization, Methodology, Writing - review & editing. **Marthe T.C. Walvoort:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Paul de Vos:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration. **Alexandra M. Smink:** Conceptualization, Methodology, Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.carbpol.2020.116863>.

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